RESEARCH NOTE

HIGH PREVALENCE OF *PFCRT* K76T MUTANTS AMONG *PLASMODIUM FALCIPARUM* ISOLATES FROM SABAH, MALAYSIA

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Abstract. Chloroquine (CQ) remains the first line drug for the prevention and treatment of malaria in Malaysia in spite of the fact that resistance to CQ has been observed in Malaysia since the 1960s. CQ-resistance is associated with various mutations in *pfcrt*, which encodes a putative transporter located in the digestive vacuolar membrane of *P. falciparum*. Substitution of lysine (K) to threonine (T) at amino acid 76 (K76T) in *pfcrt* is the primary genetic marker conferring resistance to CQ. To determine the presence of T76 mutation in *pfcrt* from selected areas of Kalabakan, Malaysia 619 blood samples were screened for *P. falciparum*, out of which 31 were positive. Blood samples were collected on 3 MM® Whatman filter papers and DNA was extracted using QIAmp DNA mini kit. RFLP-PCR for the detection of the CQ-resistant T76 and sensitive K76 genotype was carried out. Twenty-five samples were shown to have the point mutation in *pfcrt* whereas the remaining samples were classified as CQ-sensitive (wild-type). In view of the fact that CQ is the first line anti-malarial drug in Malaysia, this finding could be an important indication that treatment with CQ may no longer be effective in the future.

Keywords: *Plasmodium falciparum*, chloroquine resistant, *pfcrt*

INTRODUCTION

The number of malaria cases in Malaysia is declining with the exception of selected areas in remote communities of the East Malaysia, Sabah and Sarawak (WHO, 2010). However, sporadic outbreaks of malaria still occur in non-endemic and peri-urban areas where anopheline mosquitoes still exist. In spite of the fact that most surrounding countries have adopted artemisinin-based combination therapy

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(ACT), chloroquine (CQ) has been used as the first-line antimalarial drug to treat *P. falciparum* malaria ever since the start of malaria control program in the 1960s in Malaysia (Lokman Hakim *et al*, 1996). CQ-resistance was first reported in Malaysia in 1963 (Montgomery and Eyles, 1963). Subsequently, several reports on CQ-resistance have been reported from Sabah, Sarawak and Peninsular Malaysia. (Lokman Hakim *et al*, 1996; Cox-Singh *et al*, 2003). There is mounting evidence of drug resistant *P. falciparum* in Malaysia, which not only affects malaria control efforts but in the long run may also affect mortality due to ineffectiveness of the drugs on the parasite. Although CQ-resistant *P. falciparum* have spread throughout most malaria-endemic regions worldwide, CQ remains one of the important antimalarials because of its low cost and relative safety compared to other antimalarial drugs.

In 2000, *P. falciparum* CQ-resistant transporter (*pfcrt*) gene was identified, which is localized in the digestive vacuolar membrane of the parasite (Kuhn *et al*, 2010). The substitution from lysine (K) to threonine (T) at amino acid 76 (K76T) in the pfcrt protein appears to be a primary mutation conferring resistance to CQ (Fidock *et al*, 2000). This *pfcrt* T76 mutation is a key determinant of CQ-resistance *in vitro* and *in vivo*, suggesting that the mutation may be used as a molecular marker and tool for surveillance of *P. falciparum* CQ-resistance (Djimde *et al*, 2001).

In Malaysia, a previous study has indicated 100% prevalence of *pfcrt* K76T marker in Lundu, Sarawak (Cox-Singh *et al*, 2003). Information on the level of *P. falciparum* to currently used drugs in the country is vital to generate usable data to be used by the National Malaria Control Program in their strategic planning. Thus, the aim of the present study was to determine the presence of *pfcrt* K76T mutation in 31 *P. falciparum*-positive samples originating from individuals in the general population of Kalabakan Tawau, Sabah, Malaysia.

MATERIALS AND METHODS

Study area

The present study was confined to Kalabakan, which is located in the Tawau Division of Sabah, Malaysia. Tawau was selected as study site because it has the highest number of malaria cases reported in Malaysia in 2007 with 964 cases and 122 cases in January to February 2008 (Vector Borne Disease Control, 2008, personal communication). The study was conducted from June 2008 to October 2009 involving a population recruited from palm oil and rubber plantations as well as road construction workers.

Ethics approval and study design

The study documents were reviewed and approved by the Research Review Committee of the Institute for Medical Research and the Medical Research Ethics Committee (MREC), Ministry of Health Malaysia. Study population constituted individuals from the selected study sites. They were given written informed consent prior to participation in the study. The inclusion criteria were individuals aged 6 years or older and a positive malaria diagnosis for *P. falciparum* monoinfection. Consenting individuals were screened for malaria infection by finger prick blood diagnosis using a rapid diagnostic test kit (Paramax-3™, Zephyr Biomedicals, India).

Sample collection

Individuals positive for *P. falciparum* monoinfection were selected for venous blood collection. One hundred l aliquot of blood was spotted onto 3MM® Whatman (Brentford, United Kingdom) filter

paper. The filter paper was allowed to dry completely, transferred into individual plastic bags, labelled, and transported to the Institute Medical Research, Kuala Lumpur, Malaysia. At the laboratory, the blood-spotted filter papers were stored at room temperature in a desiccator containing silica gel until further processing. The individuals with positive malaria parasite were clinically managed in accordance with the current national policy for treatment of malaria by the health department staff.

Detection of *pfcrt* mutation using restriction fragment length polymorphism (RFLP)

Parasite genomic DNA was extracted from blood-spotted filter papers by using QIAmp® DNA Mini Kit (QIAmp; QIAGEN, Hilden, Germany), according to the manufacturer's instructions (dried blood spots protocol) with the only modifications being adjustments of the elution buffer volume used to elute DNA.

The first round PCR was performed using TCRP1 and TCRP2 primers described by Djimde *et al* (2001) with minor adjustments to the concentrations of the reagents used for the PCR reaction. In brief, 50 ng of genomic DNA, 1x PCR buffer (BioRad, Hercules, CA), 2.5 M $MgCl_2$, 200 M of each dNTP, 2.5 U of *Taq* DNA polymerase (BioRad) and 0.2 M of each primer were used in a final volume of 50 l. Three microliter aliquot of the PCR amplicon was then used in the second PCR using primer CRTD 1 and 2 in a final volume of 50 l reaction mixtures containing 1x PCR buffer (Bio-Rad), 2.0 M $MgCl_2$, 200 M of each dNTP, 2.5 U of *Taq* DNA polymerase (Bio-Rad) and 0.2 M of each primers yielding an amplicon of 145 bp. An aliquot of 7.9 l from the PCR solution was incubated with *Apo I* (New England Biolabs, Beverly, MA) at 50ºC according to manufacturer instructions in a 10 l final reaction volume. *Apo I* cleaves the wild-type allele into 111 and 34 bp fragments but does not digest K76T allele. The digested products were analyzed using Agilent 2100 Bioanalyzer and Agilent DNA 1000 Kit (Agilent Technologies, Molecular Probes, Eugene, OR) conducted according to manufacturer's instructions. All PCRs were performed using an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). DNA from CQ-sensitive 3D7 and CQ-resistant K1 *P. falciparum* strains were used as positive controls. Water was used to replace the DNA template in the PCR reaction for negative control.

RESULTS

Twenty-three sites in Kalabakan, Tawau, were included in the study. A total of 619 individuals were screened for malaria infection. Fifty-eight (9.4%) [95% confidence interval (CI) 7.07-11.67] tested positive for malaria of which 31 (5.0%) were positive for *P. falciparum*.

RFLP-PCR was performed on all the samples. The first round PCR using TCRP1 and TCRP2 primers served as template in a nested PCR using CRTD1 and CRTD2 primers to yield a 145 bp band (Fig 1). Upon digestion with *Apo I*, the wild-type codon was cleaved into 111 bp and 34 bp fragments. Of 31 samples, 25 (80.6%) were undigested by *Apo I* indicating that the samples have a point mutation at codon 76 (Djimde *et al*, 2001; Cox-Singh *et al*, 2003).

DISCUSSION

The presence of the K76T mutation gene may not necessarily mean that *P. falciparum* resistant to CQ treatment. This situation has been observed in many studies where the presence of *pfcrt* K76T mutation were detected in *in vivo* CQ

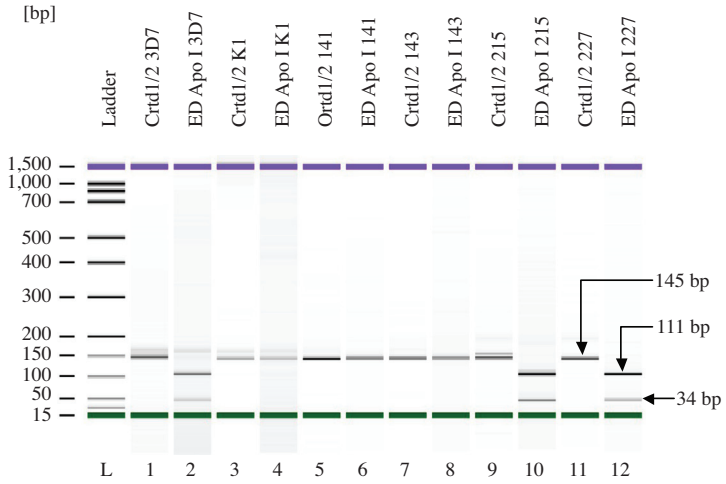

Fig 1–Representative bioanalyzer gel of *Apo* I digestion of *pfcrt* amplicons containing codon 76 polymorphism. Lanes 1, 3, 5, 7, 9 and 11 are amplicons using CRTD1 and CRTD2 primers which give a 145 bp band. *Apo I* digests wild-type amplicon into 111 and 34 bp fragments. Amplicons in lanes 2, 10 and 12 are completely digested by *Apo I* suggesting that samples 215, 227 and 3D7 (which served as CQ-sensitive control) have wild-type allele. The amplicons in lanes 4, 6, and 8 are not digested, indicating presence of mutant allele. Lane L is molecular size markers.

responder individuals, suggesting that the presence of *pfcrt* K76T mutation may not necessarily lead to treatment failure (Thomas *et al,* 2002; Vinayak *et al,* 2003). Even though there are still individuals responding to CQ treatment, the presence of K76T mutation can be considered as an early warning for CQ-resistance to happen (Vinayak *et al,* 2003).

Mutant *pfcrt* is associated with or considered to be the key marker for CQ-resistant *P. falciaprum* and this has been validated by many studies throughout the world, to name a few, Sudan (Babiker *et al,* 2001), Cameroon (Basco and Ringwald, 2001), Thailand (Lopes *et al,* 2002), Cambodia (Lim *et al,* 2003) and Sarawak, Malaysia (Cox-Singh *et al,* 2003). In Thailand, *pfcrt* mutation has been reported in 99.1% of *P. falciparum* collected from areas along Thai borders, including Thai-Malaysia border (Rungsihirunrat *et al,* 2009).

At a time of emerging resistance to even the most recent anti-malarial drugs, Malaysia is in the fortunate position that even CQ has remained relatively effective for a long time. CQ is still used as the first line antimalarial drug in Malaysia, even though there have been earlier reports on CQ-resistance in *P. falciparum* (Lokman Hakim *et al,* 1996; Cox-Singh *et al,* 2003). At present there is little information on the clinical and *in vitro* sensitivity of falciparum malaria to CQ in Malaysia. The present study showed the presence of *pfcrt* K76T mutation in 81.0% of *P. falciparum* field isolates in Sabah suggesting a high prevalence of CQ-resistance in this region. Even if our findings are not representative for *P. falciparum* CQ-resistance situation for the whole country, it is worthy to note the presence of *pfcrt* K76T mutation in the study area. This is reason for caution.

Our study population was recruited from individuals living in malaria endemic areas, who claimed not to have received any antimalarial treatment before. The presence of CQ-resistant *P. falciparum* nevertheless is likely to be attributed to drug pressure due to excessive use of CQ in Malaysia as the first line antimalarial drug for almost 50 years. Our study provides evidence for a high prevalence of *pfcrt* K76T mutation, which is known to confer CQ-resistance in *P. falciparum.* However, further study is required to obtain more

information on this gene in relation to the level of susceptibility of *P. falciparum* to CQ in Malaysia.

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